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13. ABSTRACT ***(Maximum 200 Words)***

The thrust of the work that has been done for this reporting period, is that elastin has the capacity to induce the growth of breast carcinoma cells which express galectin-3. The data suggest that elastin interacts with the cells via galectin-3 on the cell surface. Elastin was also found to be a chemoattractant for breast carcinoma cells which express galectin-3 and hence there exists an elastin-galectin-3 chemotaxis signaling pathway. Elastin and galectin-3 bind with micromolar affinities comparable to binding affinities between galectin-3 and asialofetuin or polylactosamine residues.

Overall in the 3 years that I have been funded, we have clearly demonstrated that the interaction of breast carcinoma cells with elastin, is not just a coincidence but has far reaching physiological implications that require a more rigorous study. I have currently assembled a team of molecular biologist and molecular biophycists/chemists to address this important question. If we can fully understand the role of galectin-3 in the interactions between breast carcinoma cells and elastin, then we will be able to halt the growth of breast metastatic tumors in the lung.

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Introduction. The interactions of breast carcinoma cells with extracellular matrix (ECM) proteins such as laminin, collagen IV and fibronectin are well described in the literature. However, the interactions of these cells with elastin, the dominant ECM protein in the lung, is poor described. Lung is a favored site for the metastatic spread of breast cancer. Using a galectin-3 expressing and a null expressing model, we have demonstrated for a number of years now, that galectin-3 is critical for the interactions of breast carcinoma cells and elastin. Galectin-3 is a member of the beta-galactoside binding proteins. It is a chimeric type galectin with a globular carbohydrate recognition domain (CRD) and a collagen-like domain. Galectin-3 can be expressed on the surface of cells even though it lacks a classical signal peptide. On the cell surface it can modulate the interaction of cells with extracellular matrix proteins in several ways. It modulates the spreading of the cells, the affinity of integrins for extracellular matrix proteins, and as we show here, the adhesion of cells to elastin fibers. We and others have shown galectin-3 to be highly expressed by metastatic breast carcinoma cells. The current working model is that galectin-3 is part of an adhesive complex on the surface of tumor cells which includes the 67 kDa laminin/elastin receptor protein. We hereby show that galectin-3 interacts with elastin with binding constants in the micromolar range. We also demonstrate that cells which express galectin-3 respond to chemotactic peptides emanating from insoluble elastin. We further demonstrate that cells which express galectin-3 grow more favorably on insoluble elastin but not on elastin dissolved by organic solvents. Such interactions may influence the propagation of the tumor cells in elastin rich tissues and as such of great interest in understanding the propensity of breast tumor cells to colonize the lung.

Body:

The following are the research activities that have been carried out in the laboratory since the October report of 2001. For the past one year, our goal has been to complete task # 3. Having established the binding interactions of galectin-3 with the chemotactic domain of galectin-3, we wanted demonstrate whether galectin-3 could be the key cell surface molecule that transmits chemotactic signals to the cell. To do this, we coated the bottom wells of a Boyden chamber chemotactic assay with fibrillar elastin. Galectin-3 expressing (11-9-14) and null (BT-549) expressing cell lines were then added to the top wells and their ability to crawl towards the bottom wells, lodging on the underside of the membrane which separated the upper from the lower wells determined. The galectin-3 expressing 11-9-1-4 cells moved much faster towards elastin (chemotaxis) than BT-549 (Figure 1). This is the first time that we have directly implicated galectin-3 in the chemotaxis of breast carcinoma cells towards elastin. We are in the process of doing more detailed experiments to determine which other elastin peptides if any, can enhance the chemotaxis of the cells. We next questioned whether or not galectin-3 expressing cells could grow more rapidly on both soluble and insoluble elastin. We observed that only insoluble elastin fibers were capable of supporting growth of breast carcinoma cells.

The galectin-3 expressing lines had accelerated growth rate on elastin compared to the null expressing cell line (Figure 2).

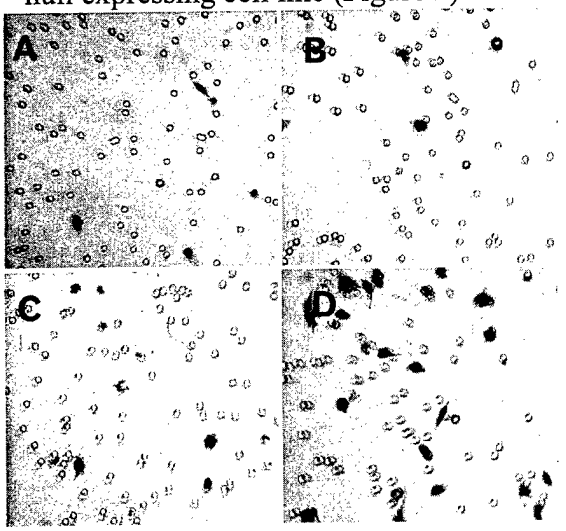


Fig. 1. The bottom wells of a chemotactic chamber were either coated with BSA (A,B) or insoluble elastin (C,D). The breast galectin-3 null expressing BT-549 were added to the top wells (A and C) while the galectin-3 expressing 11-9-1-4 were added to (B and D). The cells were allowed to adhere to the wells for 6 hours after which cells from the top wells were removed by cotton swabs. The cells underneath the membranes were stained,

The elastin induced growth of breast carcinoma cells is interesting for one main reason. Breast carcinoma cells which metastasize to elastin tend to multiply very rapidly if the conditions are right and they escape from dormancy. Our studies so far point to the elastin in the lung as a major growth stimulator. However the cells which respond to stimulatory activities of elastin are those which also express high levels of galectin-3. As a cautionary note, we have to be very careful when interpreting these data because there are many variables to be controlled and that is the reason why these experiments are so difficult to design.

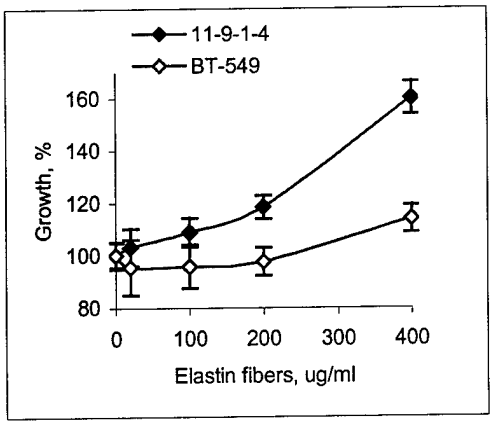
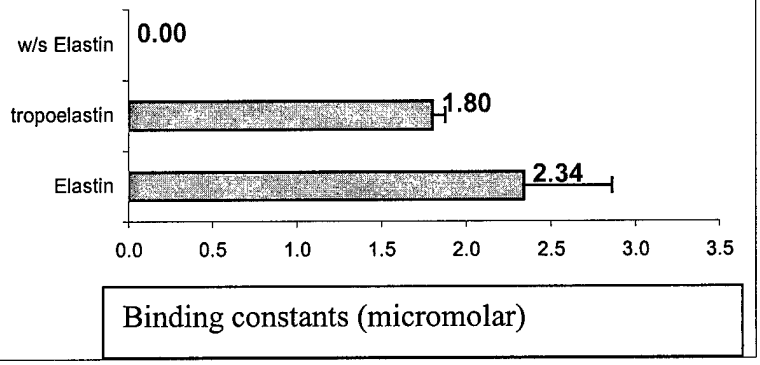


Fig. 2. The growth of breast carcinoma cells on elastin coated wells. The well were coated with elastin fibers (0-400 μg/ml). The non specific sites were blocked with BSA and the cells added to the wells (20,000/well). The cells were allowed to grow for three days at 37°C . At the end of the third day, medium was replaced with fresh medium and Alamar blue added and the proliferative potential of elastin on the cells determined.

The most convincing experimental proof that any given pair of proteins has a binding interaction between them is to determine their binding constants. We have asserted in this progress report and previous reports that galectin –3 appears to have strong binding interaction with elastin. We therefore used spectrophotometric approaches to accurately

determine the binding parameters between elastin and galectin-3 (Figure. 3) The reason why it has taken us such a long time to do this determination is that in the past we relied on elastin solubilized by organic solvents. These have not given us consistent data. However the use of fine elastin particles , gave us very reproducible binding constants that were comparable to those obtained from the interaction between galectin-3 and soluble tropoelastin (Figure 3).

Fig. 3. The binding constants of tropoelastin and particulate elastin to galectin-3. The constants are comparable to those between galectin-3 and asialofetuin. They are in the micromolar range.



The most interesting observation in this study was that the binding constants between galectin-3 and elastin were very comparable to those between galectin-3 and glycans such asialofetuin and polylactosamine residues.

Future Direction. I am now in the process of writing an RO1 grant proposal to enable me to continue this work. We will continue to explore the proliferative effects of elastin on breast carcinoma cells. We deem this to be a very important area of concentration which will allow us to understand the likelihood of metastatic breast cancer cells to metastasize to the lung and multiply there. Also we will do more detailed studies on the chemotactic properties of elastin and elastin peptides. We will ask questions such as, are there other chemotactic peptides apart from VGVPAG which can be released from elastin by tissue specific elastases?

KEY RESEARCH ACCOMPLISHMENTS.

- Demonstrating that insoluble elastin is a chemoattractant for breast carcinoma cells expressing galectin-3 is a milestone in our research.
- The use of state of the art spectrometric approaches to determine the binding constants between galectin-3 and elastins is a key accomplishment.

REPORTABLE OUTCOMES.

We presented an abstract at the era of Hope meeting in Orlando Florida. We are currently preparing a manuscript to be considered for publication in Experimental Cell Research.

CONCLUSIONS. After studying the interactions between breast carcinoma cells and elastin for the past three years, this is the time to pause and ask the 'so what?' question. Has this been just an academic exercise or do we see some tangible outcomes down the line? At the end of the 3 year period we are left with more questions than answers which is a good thing in research. It is good because we need to do more to be sure that this is an important area of investigation which should set new paradigms in breast cancer research. The take home lesson is that the interaction of breast carcinoma cells with elastin is not just a coincidence or of no consequence. Elastin fibers influence breast carcinoma cells and perhaps other cell types in the extracellular milieu more or less to the same extent as collagen or laminin regulates the differentiation or dedifferentiation states of breast cancer in vivo. The challenge for this research effort is to determine how elastin transmits its growth and chemotactic signals to breast and other carcinomas.

NOTE: This is written as an annual report because we have asked for a no cost-extension on the grant to enable me to complete task # 3. Specifically experiments are ongoing to determine whether elastin mediated signaling alters the adhesion of breast carcinoma cells to other extracellular matrix proteins.

Publications from the Grant.

1) Zhu W.Q., and Ochieng, J. Rapid release of Galectin-3 from breast carcinoma cells by fetuin. Cancer Res. 61, 1869-1873, 2001

2) Ochieng, J., Warfield, P., Green-Jarvis, B., and Fentie, I. Galectin-3 regulates the adhesive interaction between breast carcinoma cells and elastin. J. Cell. Biochem. 75, 505-514, 1999.

I n preparation.

1)Ochieng, J., Lukyanov, P., and Furtak, V. Interactions of Breast Carcinoma Cells with Elastin. To be submitted to Experimental Cell Research. (2002).

APPENDICES.

Journal of Cellular Biochemistry 75:505–514 (1999)

Galectin-3 Regulates the Adhesive Interaction Between Breast Carcinoma Cells and Elastin

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Abstract Galectin-3 is a beta-galactoside binding lectin whose precise physiological role is not yet defined. In the present studies, we questioned whether galectin-3 plays a role in the adhesion of breast carcinoma cells to elastin. The impetus for this analysis was the initial observation that the cellular receptor for elastin, the 67 kDa elastin/laminin protein may have galectin-like properties (Mecham et al. [1989] J. Biol. Chem. 264:16652–16657). We therefore analyzed the adhesion of breast carcinoma cells to microtiter wells coated with elastin under conditions which eliminate integrin participation in adhesion. The adhesion assay was done in the absence and presence of purified recombinant galectin-3. We hereby demonstrate that high concentrations of galectin-3 ligate breast carcinoma cells to microtiter wells coated with elastin. Galectin-3 also demonstrated a specific binding interaction with purified elastin in a dose and lactose dependent manner. Furthermore we demonstrated by immunoprecipitation that endogenous galectin-3 in breast carcinoma cells is associated with tropoelastin. Lastly, the breast carcinoma cells which expressed galectin-3 on their surface, demonstrated enhanced cellular proliferation on elastin compared to galectin-3 null expressing cells. These studies suggest that galectin-3 is capable of regulating the interactions between cells and elastin. J. Cell. Biochem. 75:505–514, 1999.

Key words: galectin-3; elastin; ligation; breast; carcinoma

Whereas the precise physiological role of galectin-3 is still elusive, mounting evidence suggests its involvement in the regulation of cell-extracellular matrix interactions [Sato and Hughes, 1992; ochieng et al., 1992; Warfield et al., 1997; Kuwabara and Liu, 1996]. In breast epithelial cells, low expression of galectin-3 has been linked to low cloning and plating efficiencies while high expression improves the interaction of cells with different substrata [Makker et al., 1995; Warfield et al., 1997]. The mechanisms by which galectin-3 modulates the interactions of cells with extracellular matrices is not well defined, and may vary in different cell types and at different stages of differentiation or transformation. For example, it has been demonstrated that galectin-3 expression is crucial for transformed cells to acquire the anchorage independent growth in soft agar [Raz et al., 1990; Makker et al., 1995]. Recent studies suggest that galectins modulate cell to extracellular matrix interactions in a novel fashion by interaction with integrins such as $\alpha 7\beta 1$ or $\alpha 1b1$ [Gu et al., 1994; ochieng et al., 1998].

It is presumed that cells interact with elastin via the non-integrin 67 kDa elastin/laminin receptor [Lesot et al., 1983; Malinoff and Wicha, 1983; Rao et al., 1983]. Whereas the mature receptor on the cell surface is 67 kDa, its full-length gene encodes only a 37 kDa precursor protein [Yow et al., 1988; Rao et al., 1989]. It is believed the precursor is bonded to a ~30 kDa protein to form the mature chimeric receptor. Since the discovery of this receptor, it has been known that it has galectin-like properties because lactose modifies its biological activities. More recently, the work of Buto et al. [1998], suggested that the 67 kDa receptor is a heterodimer stabilized by strong intramolecular hydrophobic interactions, carried by fatty acids bound to the 37 kDa precursor and to a galectin-3 cross-reacting molecule. We have therefore hypothesized that galectin-3 is part of the 67 kDa elastin/laminin receptor complex

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and contributes to the cell-elastin or cell-laminin interactions.

On the surface of mesenchymal cells, there are high affinity receptors for elastin-peptides. These peptides are capable of triggering intracellular signaling which may be responsible for synthesis or recruitment of cell surface ~120 kDa receptors for insoluble elastin [Groult et al., 1991]. It is possible that similar signaling responses may be triggered in non-mesenchymal cells. Breast carcinoma cells interact well with insoluble elastin [Parsons et al., 1991], and the elastin receptors in these cells may be activated in a fashion similar to the mesenchymal cells. Despite recent progress in the characterization of the 67 kDa elastin receptor, our knowledge of cell-elastin interactions still lag behind the well characterized cell-laminin or cell-collagen interactions. It is likely that in regards to tumor growth either in situ or in micrometastases, cell-elastin interactions are as important as the interactions of cells with either laminin or collagen IV.

We hereby demonstrate that galectin-3 at high extracellular concentrations, can ligate breast carcinoma cells to elastin. We further demonstrate that galectin-3 is tightly associated with insoluble elastin, an association which can be downregulated by lactose. Our data also suggest that galectin-3 is associated with soluble tropoelastin intracellularly and is an integral part of the cell to elastin receptor complex.

MATERIALS AND METHODS

Human breast carcinoma cell lines BT-549; Sk-Br-3; MDA-MB-435; and 11–9-1–4 (derived from BT-549) [Makker et al., 1995] were obtained from Dr. Avraham Raz, Karmanos Cancer Institute. All the cell lines were cultured in DMEM (Sigma) supplemented with 100 µg/ml penicillin-streptomycin, 2.5 µg/ml Fungizone, 20 ng/ml epidermal growth factor, 98 ng/ml cholera toxin, 10% heat inactivated fetal bovine serum, 2 mM glutamine, and non-essential amino acids. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Recombinant galectin-3 was isolated and purified as described [Ochieng et al., 1993].

Ligation of Breast Carcinoma Cells to Elastin by Recombinant Galectin-3

Insoluble elastin was washed extensively with PBS containing 1 M NaCl to dissociate any non-specifically bound protein. The elastin fibers were then resuspended in PBS at 4 mg/ml and the suspension (100 µl/well) added to the wells of an ELISA micro-titer plate (Immulon 1B, Dynex Technologies) and incubated overnight at 37°C. The wells were then washed once with PBS and 200 µl of 3% heat inactivated fatty acid free bovine serum albumin in PBS added to each well to block non-specific sites for 1 h at 37°C. The wells were once more washed with PBS and serumless, calcium-free DMEM/F12 medium containing increasing doses (0–25 µg/well) of galectin-3 added to each well and allowed to incubate for 15 min at 37°C. The breast carcinoma cells, BT-549 (subclone 11–9-1–4) cells were then added to the wells at 5×10^4 cells/well in the serumless medium and allowed to incubate for 1 h at 37°C. The same number of cells/well were also plated in wells coated with 3% bovine serum albumin (open bars) in the presence of galectin-3 (0–25 µg/well) and incubated as above. The wells were then washed (3×) with serumless medium and the number of cells ligated to elastin per well determined by the Alamar blue method as previously described [Warfield et al., 1997].

To test whether the cells adhere to glycoproteins which may be associated with elastin fibers such as fibrillin, the elastin (5 mg/ml) was treated with anhydrous hydrazine and 1% hydrazine sulfate overnight at 80°C. The solubilized elastin was then used to coat the wells of a microtiter plate and cellular adhesion performed as described above.

Growth of Breast Carcinoma Cells on Elastin

Insoluble elastin was washed and added to ELISA microtiter plates as described above. Breast carcinoma cells (galectin-3 expressing and null expressing cell lines, 2×10^4 cells/well) were then added to wells containing elastin after blocking with BSA, in DMEM/F12 medium containing 10% serum and allowed to grow for up to 10 days in a humidified CO_2 incubator. The cells were then photographed using a digital camera (Kodak) and the images analyzed by Adobe Photoshop.

Binding of Galectin-3 to Elastin

Insoluble elastin was washed with high salt and added to ELISA microtiter plate as described above. After blocking the non-specific sites with 3% bovine serum albumin, serially diluted aliquots of recombinant galectin-3 (0–16 µg/well) were added to the wells in duplicates

in the absence and presence of 200 mM lactose. To demonstrate elastin binding specificity, galectin-3 in the same concentration range was also added to wells coated with BSA alone. After 1 h of incubation at 37°C, the wells were washed twice with PBS containing 0.05% tween and 100 µl of rabbit polyclonal antibodies to galectin-3 (1:500) added to each well and incubated at 37°C for 30 min. The wells were then rinsed 3× with PBS and 50 µl of biotinylated goat anti-rabbit IgG (Vectastain-ABC kit) added to each well and incubated for 5 min at 37°C. After washing the wells (3×) with PBS, 50 µl of Vectastain ABC reagent was added to each well and incubated for a further 5 min at 37°C. The wells were then washed extensively (6×) with PBS and then alkaline phosphate substrate (Blue PhosTM) added and absorbance (650 nm) determined by a microplate reader (Dynex Tech.).

Immunoprecipitation of Elastin-Galectin-3 Complex

In order to establish the physiological relevance of elastin-galectin-3 interactions, we questioned whether the two proteins are associated either intracellularly or on the cell surface of breast carcinoma cells which express both galectin-3 and elastin. The cells were lysed in lysis buffer (calcium/magnesium free PBS containing, 0.5% NP 40, 1 mM EDTA, 2 mM PMSF, pH 7.5). The lysate was pre-cleared by incubating for 2 h with protein A/G agarose and aliquots (200 µg of protein each) of the lysate incubated with 0, 5, 10, and 20 µg of recombinant galectin-3 for 2 h at 4°C followed by either rabbit pre-immune serum or rabbit polyclonal anti-galectin-3 antibodies (10 µg/tube) for 10 h at 4°C in eppendorf tubes. Protein A/G agarose was then added to each tube and allowed to incubate for 2 h at 4°C. The agarose was then washed 5× with PBS containing 0.05 % tween and boiled for 5 min with 1X SDS-sample buffer. The samples were then analyzed by 10% SDS-PAGE, transferred to immobulin-P membrane and probed with mouse anti-human elastin which recognizes bands of tropo-elastin in Western blots. The membranes were washed and probed with peroxidase labeled sheep anti-mouse IgG, followed by incubation with Amhersham chemiluminescence reagents and exposed to X-ray film. In another set of experiments, the lysates (200 µg of protein each) were incubated with either non-immune mouse antibodies or mouse monoclonal anti-elastin (Sigma) after pre-clearance. The immunoprecipitation was done as described above, and the membranes probed with rat monoclonal anti-galectin-3 (TIB 166) [Warfield et al., 1997]. The membranes were washed and then probed with peroxidase labeled goat anti-rat IgG and the bands visualized as described above.

RESULTS

Interactions of Breast Carcinoma Cells With Elastin

Strong adhesion of cells to extracellular matrix proteins depend on a number of factors, such as the activation state of the cell surface receptors and optimal biochemical conditions such as pH, divalent cations, and incubation temperatures. In the case of cell to elastin adhesion, despite the lack of involvement of integrins, divalent cations, and serum factors are needed for optimal interaction with insoluble elastin [Ochieng et al., unpublished data]. The divalent ions and serum factors may be necessary for the activation or recruitment of the 67 kDa elastin receptor. In analyzing the involvement of galectin-3 in cell to elastin interaction, we employed conditions which were unfavorable for integrins (lack of divalent ions). Biological activity of galectin-3 is independent of divalent cations and serum, especially when using exogenously supplied protein. Serum was excluded in the medium because serum proteins such as fibronectin and fetuin could potentially interact with elastin and promote cellular adhesion via other receptors.

In the adhesion assays, the insoluble elastin after washing in buffer containing high salt, was immobilized very well to the floor of the wells and could not be removed by gentle washing. We have consistently shown that galectin-3 expressing breast carcinoma cells such as 11–9-1–4 and MDA-MB-435 interact much better with elastin compared to low galectin-3 expressing cells such as SK-Br-3. In our hands, we have repeatedly observed that approximately 10–20% of the galectin-3 expressing cells when added to micro-titer wells coated with elastin will adhere within 1 h of incubation at 37°C. In the case of low galectin-3 expressing cells, less than 5% of the added cells adhere after 1 h of incubation (data not shown). This phenomenon is true for other extracellular matrix proteins, laminin, and collagen IV [Warfield et al. 1997].

We therefore wanted to determine whether exogenously added galectin-3 could improve the interaction of breast carcinoma cells with elastin. Our data (Fig. 1) demonstrate that galectin-3 by itself can significantly promote the adhesion of cells to immobilized elastin. The galectin-3 mediated ligation of cells to elastin was concentration dependent. It is likely that for cells to interact optimally with elastin, secreted galectin-3 is concentrated on the cell surface to ligate or improve their adhesion to elastin. If this is so, then galectin-3 mediated adhesion of cells to elastin could be a physiologically relevant mechanism of cell-elastin interaction. The experiment was repeated three times with BT-549 clone 11–9-1–4, and once with MDA-MB-435. Both are tumorigenic cells lines of breast cancer. In all cases, high concentrations of galectin-3 were capable of ligating cells to elastin. We estimated that approximately 40–50% of the cells added to the wells were ligated to elastin at 25 μg/ml of galectin-3. Similar concentrations of galectin-3 were unable to ligate the cells to wells coated with BSA alone (Fig. 1).

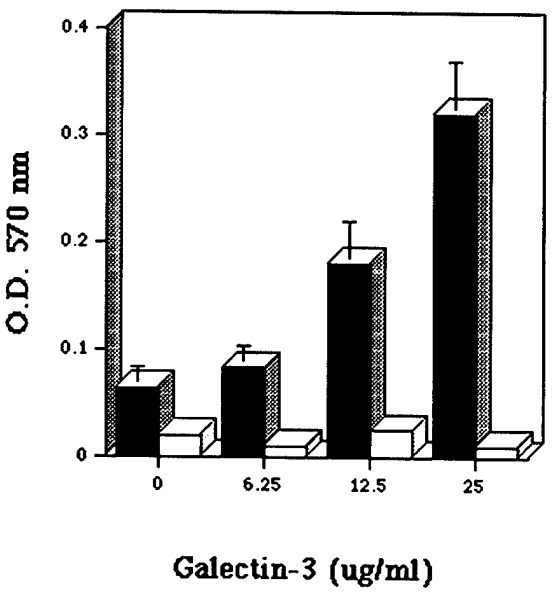

Fig. 1. The ligation of breast carcinoma, BT-549, subclone 11–9-1–4 cells to elastin by galectin-3. The cells in calcium free, serumless DMEM/F12 medium were plated at 5×10^4 cells/well in elastin coated microtiter wells (solid bars) in the presence of recombinant galectin-3 (0–25 μg/well). The same number of cells/well were also plated in wells coated with bovine serum albumin (open bars) in the presence of galectin-3 (0–25 μg/well). The adherent cells were determined after 1 h. The experiment was repeated three times with 11–9-1–4 cells.

To further explore the interaction of breast carcinoma cells with elastin, the cells were added to elastin coated wells and allowed to incubate for up to 10 days. In this experiment, we were interested in testing the hypothesis that galectin-3 expressing cells have a higher propensity to adhere and proliferate on elastin compared to low galectin-3 expressing cells. By day 3 of incubation of the various cell lines with elastin, we consistently observed that the cells (11–9-1–4) which express high levels of galectin-3 [Makker et al., 1995; Warfield et al., 1997], interacted very well with insoluble elastin (Fig. 2A). There were numerous cells (arrow heads) associated with individual elastin fibers (arrows). This was in contrast to the cells (SK-Br-3) with little or no expression of galectin-3 (Fig. 2B). In this case, there were fewer cells associated with elastin fibers and were easily detached by washing. Interestingly, the cells which had better interactions with elastin (high galectin-3 expression) also had a tendency to proliferate while on the elastin fibers or in close proximity to the fibers as shown in Figure 2A. After 7 days of growth on elastin fibers, the galectin-3 expressing cells (11–9-1–4) literally covered the elastin fibers, resulting in expanding colonies of proliferating cells (Fig. 2C). The galectin-3 null expressing cells (SK-Br-3), on the other hand, showed little or no proliferation on elastin after 7 days of growth (Fig. 2D). The experiment was repeated four times with similar results each time. The experiment was also repeated with MDA-MB-435 (high galectin-3 expression) and BT-549 (lack galectin-3 expression) cell lines. The MDA-MB-435 cells as expected proliferated rapidly on elastin fibers, while BT-549 only interacted marginally with elastin (data not shown).

Interaction of Galectin-3 With Elastin

To explain the ability of galectin-3 to ligate cells to elastin, we questioned whether the lectin interacts specifically with insoluble elastin. Indeed in five separate experiments, we demonstrated by ELISA that galectin-3 interacts very strongly with insoluble elastin. The binding curve was saturable and lactose dependent (Fig. 3). Similar concentration of galectin-3 did not bind to bovine serum albumin coated wells (nonspecific binding). To eliminate the possibility that galectin-3 interacts via the sugar moieties which may be present in elastin, the elastin

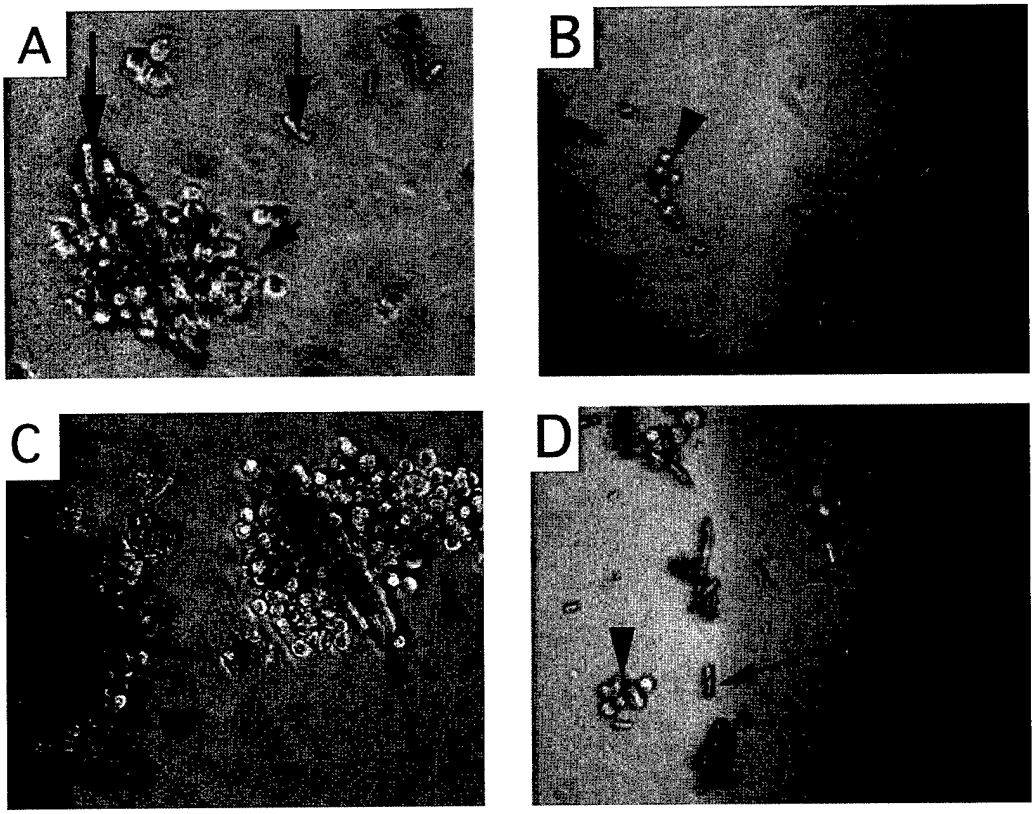

Fig. 2. Growth of galectin-3 expressing and null expressing cell lines on elastin. The wells of a microtiter plate were coated with elastin as described in Materials and Methods. The cells (2×10^4 cells/well) were then added to the elastin coated wells in DMEM/F12 medium containing 10% bovine serum and allowed to grow for 3 days (**A,B**) or 7 days (**C,D**). Galectin-3 expressing 11–9-1–4 cells (**A,C**) and SK-Br-3 (**B,D**). The cells are depicted by arrow-heads and elastin-fibers by arrows.

fibers were treated with anhydrous hydrazine which cleaves glycosidic bonds. The anhydrous hydrazine completely solubilized the elastin after about 5 h of the treatment. The addition of galectin-3 to wells coated with hydrazine solubilized elastin yielded saturable binding curves similar to Figure 3 (data not shown), suggesting that galectin-3 was directly interacting with peptide domains of elastin in a novel fashion.

To further explore the interaction of galectin-3 with elastin, we questioned whether intracellularly expressed elastin (tropoelastin) is associated with galectin-3 in cells which express both proteins. It has been demonstrated that whereas tropoelastin is mainly synthesized by fibroblasts in the mammary gland, breast epithelial cells can also express the protein [Krishnan and Cleary, 1990]. Our data clearly demonstrate that polyclonal antibodies to galectin-3 can bring down a complex of proteins containing tropoelastin (with bands in the range of ~55–70 kDa) from lysed breast carcinoma cells (Fig. 4A). Non-immune rabbit serum (control) failed to specifically immunoprecipitate the complex (Fig. 4A, lane 1). Addition of excess recombinant galectin-3 to the lysates (lanes 3 to 5) did not increase the amount of immunoprecipitable complex, implying that the complex probably exists intracellularly in a saturated state with all the galectin-3 binding sites on tropoelastin occupied. The complex was also brought down with mouse monoclonal antibodies against elastin. When the membrane was probed with rat anti-galectin-3, a strong band at 30 kDa (galectin-3) and a smaller band at ~62 kDa, were observed (Fig. 4B). The data strongly suggest that the association between galectin-3 and elastin is a physiologically relevant phenomenon. Studies are currently ongoing to evaluate further the nature of this complex, including the ~62 kDa protein which cross-reacts with anti-galectin-3 antibodies.

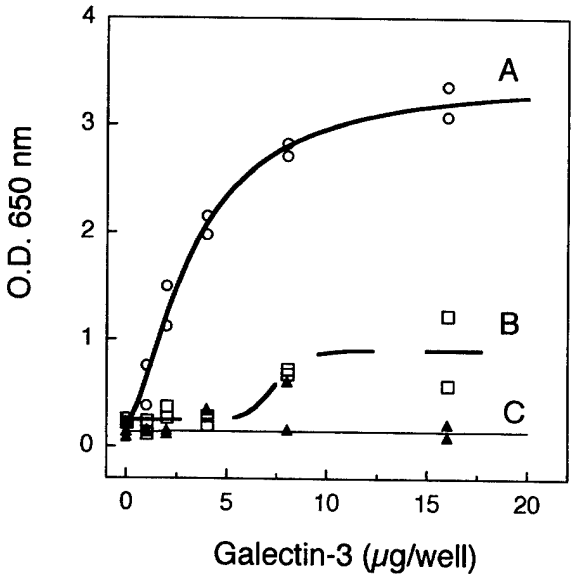

Fig. 3. Binding of galectin-3 to elastin. The wells of a microtiter ELISA plate were coated with elastin as described in Materials and Methods. Recombinant galectin-3 (0–16 μg/well) was then added to the wells in duplicates in the absence of lactose (circles; line **A**) or presence of 200 mM of lactose (squares; line **B**). Galectin-3 in the same concentration range was also added in duplicates to wells coated with bovine serum albumin alone (triangles; line **C**). After 1 h of incubation at 37°C, galectin-3 bound to elastin was determined by ELISA as described in Materials and Methods. The absorbance of the alkaline phosphate substrate was determined at 650 nm.

DISCUSSION

In the present study, we have demonstrated that galectin-3 associates in a novel fashion with both the soluble and insoluble elastin. This specific association could be important in the regulation of cell-elastin interactions. The adhesive interactions of cells with elastin is not well defined compared to the other extracellular matrix proteins, because the main physiological function of elastin is to impart elasticity to vertebrate elastic tissues. Nevertheless, it has been demonstrated that insoluble elastin as well as elastin peptides are capable of promoting not only cellular adhesion, but also proliferation [Parsons et al., 1991; Jung et al., 1998]. It is probable that in the case of certain primary as well as metastatic tumors growing in elastin rich tissues such as the lungs, their interaction with elastin is pivotal to their proliferative potential and formation of micrometastases in those tissues [Timar et al., 1991; Svitkina and Parsons, 1993]. For example, in the case of breast elastosis, interaction of the tumor cells with excess elastin in the breast may exacerbate tumor growth [Kao et al., 1986; Khatun et al., 1992].

It is established that the 67 kDa elastin/laminin receptor has galectin-like properties because lactose and anti-galectin-1 antibodies are capable of modifying its biological activities [Mecham et al., 1989]. This, taken together with the recent studies suggesting galectin-3 may be a novel component of the 120 kDa elastin/laminin receptor complex [Buto et al., 1998], prompted us to question whether galectin-3 has a direct role in cell-elastin interactions. Presently it is not clear which part of the chimeric 67 kDa receptor protein is responsible for strong cellular adhesion to elastin. Sequences in the 37 kDa precursor protein have been shown to interact with elastin peptides [Castronovo et al., 1991]. However the contribution of the ~30 kDa component in elastin binding is virtually unknown.

The ability of galectin-3 to interact with elastin in a lactose dependent manner suggests that this is a novel interaction. The interaction may involve both the R- and carbohydrate recognition domains which have been shown to participate in non-covalent homodimeriation of the molecule leading to positive cooperativity [Hsu et al., 1992; ochieng et al., 1993; Kuklinski and Probsteimer, 1998]. To explain the ability of galectin-3 to ligate the cells to elastin, we postulate that the exogenously supplied recombinant galectin-3 forms a covalent complex with the 37 kDa precursor of the 67 kDa elastin/laminin receptor on the cell surface resulting in the mature protein which in turn promotes the cellular adhesion to elastin. Alternatively high concentrations of galectin-3 may form higher order oligomers by positive cooperativity [Hsu et al., 1992; ochieng et al., 1993], with some molecules binding specifically to elastin and others to its cell surface expressed ligands such as lysosomal associated membrane proteins [Do et al., 1990], thereby acting as a bridge which links the cells to elastin coated wells (Fig. 5). Other cellular mechanisms may also be involved. For example, cell-elastin interactions may take place in two or more phases. Galectin-3 on the cell surface, may form a temporary bridge between cells and insoluble elastin, followed by the full participation of the 67 kDa in the adhesion as a late response.

We compared growth potentials of various cell lines on elastin, to further implicate galectin-3 in cell-elastin interactions. All the galec-

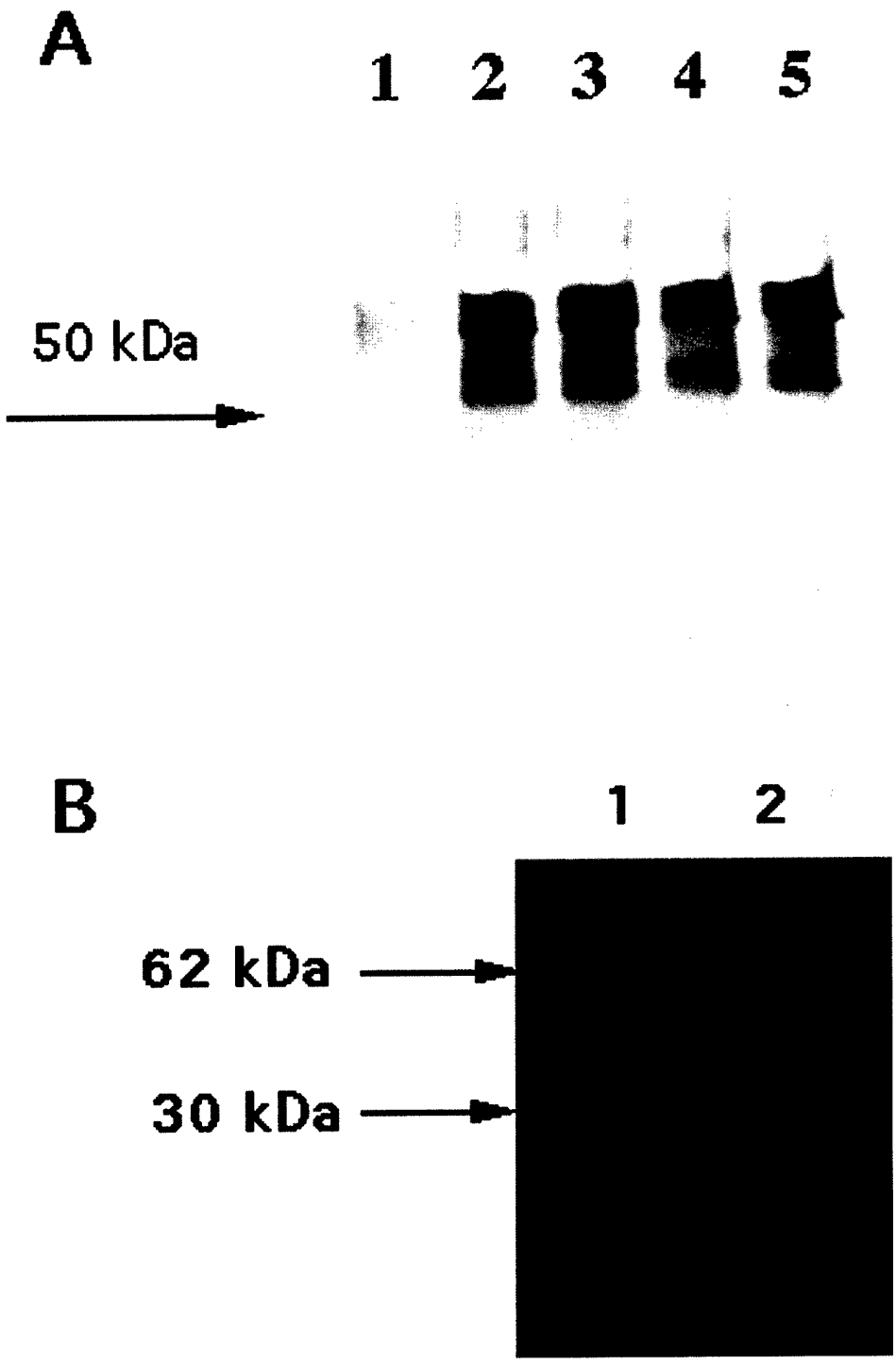

Fig. 4. Association of Elastin and galectin-3. Galectin-3 expressing breast carcinoma cells were lysed and the lysate immunoprecipitated with either rabbit pre-immune serum (**A, lane 1**) or rabbit polyclonal antibodies to recombinant human galectin-3 (**A, lanes 2–5**). In lanes 2–5, the lysates were incubated with 0, 5, 10, and 20 μg of recombinant galectin-3 for 2 h at 4°C prior to the addition of galectin-3 antibodies. The samples then resolved by 10% reducing SDS-PAGE, blotted, and the membrane probed with mouse monoclonal antibodies to elastin. The bands represent tropoelastin ~62–67 kDa. The immunoprecipitation was also done with either non-immune mouse serum (**B, lane 1**) or mouse monoclonal anti-elastin (**B, lane 2**). In this case the membrane was probed with rat monoclonal anti-galectin-3. The galectin-3 band (30 kDa) as well as a band which most likely represents a galectin-3 dimer (~62 kDa) are represented in **B, lane 2**.

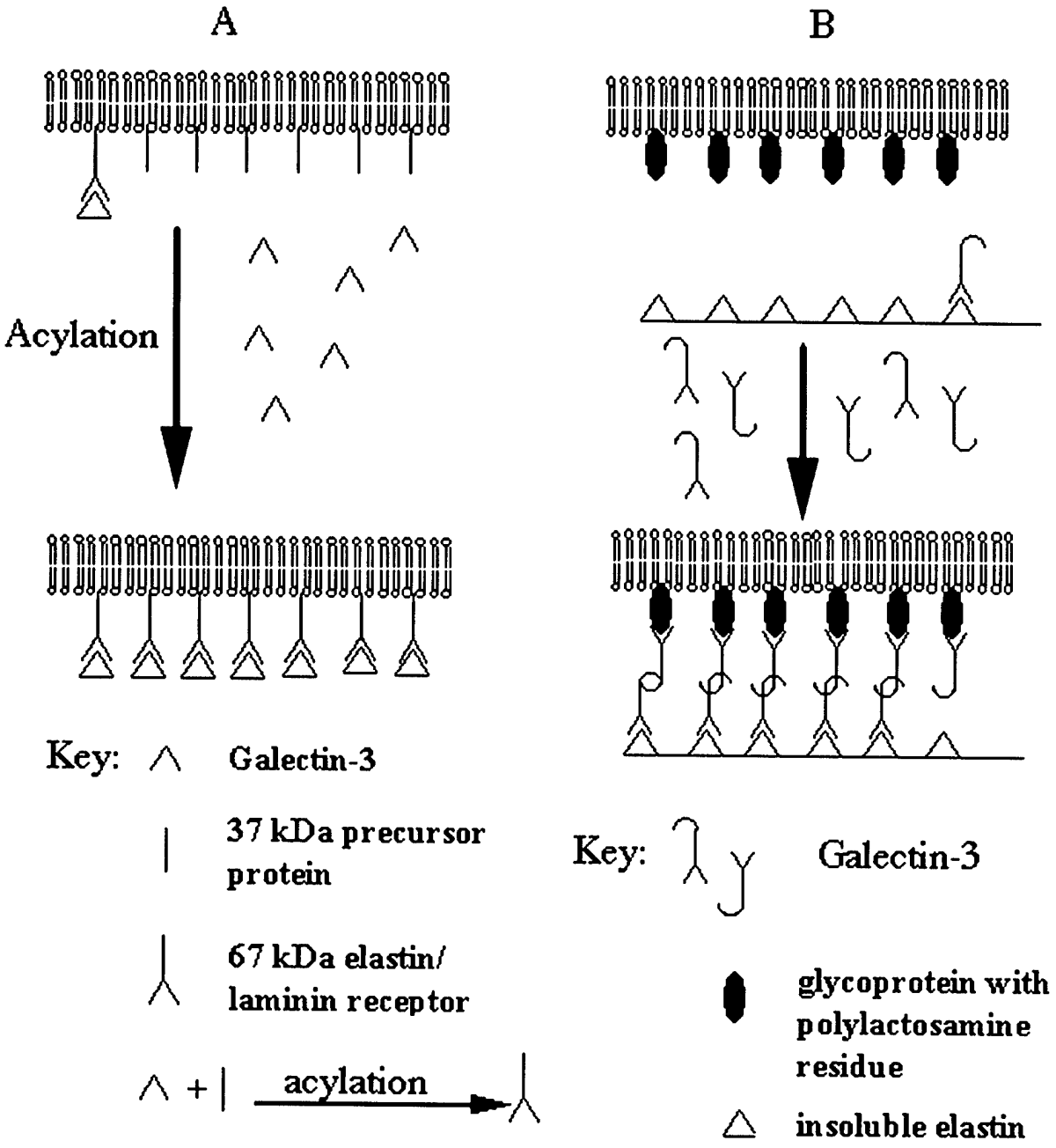

Fig. 5. A model depicting two possible ways by which galectin-3 ligates cells to elastin. In **A**, galectin-3 and the precursor of 67 kDa laminin receptor are joined by acylation to form the mature 67 kDa laminin receptor which is then used for adhesion to elastin. In **B**, some of the galectin-3 molecules interact with elastin while others with polylactosamine containing glycans on the cell surface via their carbohydrate recognition domains. The galectin-3 molecules then interact with each other via their R-domains, forming bridges which ligate the cells to the elastin.

tin-3 expressing cell lines proliferated rapidly on elastin while the galectin-3 null expressing cell lines only interacted marginally with elastin. Presently we do not know whether galectin-3 expression is the rate limiting step in such interactions. One approach to address this quandary is to stably transfect the galectin-3 expressing cell lines with anti-sense galectin-3 gene and show whether the transfected cells have reduced proliferative capacity on elastin. Galectin-3 expression has already been demonstrated to play a significant modulatory role in the interaction between cells and two extracellular matrix proteins, laminin and collagen IV [Warf-

ield et al., 1997]. Interestingly, the 67 kDa elastin/laminin receptor can also mediate the interaction of cells with collagen [Minafra et al., 1992].

The ability of anti-galectin-3 polyclonal antibodies to immunoprecipitate a complex consisting of tropoelastin, suggests that galectin-3 is tightly associated with the intracellular soluble elastin. Presently, we do not know the significance of this association. It could be a novel pathway by which galectin-3 is secreted to the extracellular space since this protein lacks the signal peptide [Barondes et al., 1994]. We cannot however, rule out the possibility that the complex also comprises the 67 kDa receptor which has been shown to be associated with tropoelastin [Hinek and Rabinovitch, 1994]. Therefore the association of galectin-3 and intracellularly expressed tropoelastin and possibly the insoluble extracellular elastin implies a modulatory role in cell to elastin interactions. Moreover this modulatory role of galectin-3 may extend to the interaction of cells with other extracellular matrix proteins such as laminin [ochieng and Warfield, 1995].

In summary, we have demonstrated that galectin-3 on the cell surface has the propensity to ligate or increase the adhesion of breast carcinoma cells to insoluble elastin. We have also demonstrated that galectin-3 interacts specifically with both soluble and insoluble elastin. It appears that galectin-3 uses its carbohydrate recognition domain in its interaction with elastin. The enhanced interaction of breast carcinoma cells (which express galectin-3) with elastin, further implicates galectin-3 in cell to elastin interactions. We also postulate based on our data, that galectin-3 may cooperate with the 67 kDa elastin/laminin receptor protein or its precursor to regulate cell-elastin interactions. Furthermore, it is tempting to speculate that the expression of galectin-3 and the 67 kDa elastin-laminin receptor confer on breast carcinomas, the propensity to form micrometastasis on elastin-rich tissues such as the lungs.

ACKNOWLEDGMENT

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Rapid Release of Intracellular Galectin-3 from Breast Carcinoma Cells by Fetuin[1]

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ABSTRACT

Galectin-3, a β-galactoside binding protein, plays a significant role in cell to extracellular matrix interactions. Despite its extracellular expression, the precise physiological mechanisms that trigger its release from the intracellular milieu have not been characterized. The present analyses were, therefore, done to identify the extracellular matrix proteins with propensity to induce the release of intracellular galectin-3 from breast carcinoma cells. Our studies demonstrate that fetuin, a serum glycoprotein that is abundant in the fetal serum, is capable of inducing the rapid release (~1 min) of intracellular galectin-3 from the cells. The mechanism by which galectin-3 is rapidly released appears to be novel and does not depend on changes in intracellular calcium levels. We also report that galectin-3-expressing breast carcinoma cells in serumless medium adhere and spread well on microtiter wells in the presence of fetuin and divalent ions in a carbohydrate-dependent manner. The data suggest that fetuin is a natural modulator of galectin-3 secretion/release and that the secreted galectin-3 modulates the activity of cell surface receptors for extracellular matrix proteins.

INTRODUCTION

Galectins are a growing family of carbohydrate-binding proteins that share affinity for β galactosides and significant sequence homology in their carbohydrate-binding domains (1–3). Galectin-3 is expressed in the nucleus, in the cytoplasm, and on the cell surface of most epithelial cells and can be secreted into the extracellular matrix (4). On the cell surface, galectin-3 plays critical roles in cell-cell or cell-extracellular matrix interactions. It has been shown to mediate homotypic aggregation that may be responsible for tumor emboli (5, 6). It has also been shown to be responsible for rapid adhesion of breast carcinoma cells to ECM[3] proteins such as collagen IV, laminin, and elastin (7, 8).

Galectin-3 like many other cytosolic proteins, such as thioredoxin (9), interleukin-1β (10), and acidic and basic fibroblast growth factor (11), can traverse the plasma membrane and yet lacks signal peptides necessary for secretion via the classical secretory pathway. The mechanisms by which galectin-3 and other proteins that lack signal peptides are secreted via the nonclassical pathway have yet to be elucidated. Galectin-3 may be concentrated in secretory vesicles that are concentrated in the membrane domains (12). It has been shown that NH_2-terminal domain of galectin-3 is critical for its secretion and is the driving force that localizes it in the secretory vesicles (13, 14, 15). How galectin-3 moves from these vesicles into the ECM or the mechanism that triggers the exocytosis of these vesicles is the gap in our current knowledge.

It has been demonstrated that whereas secretion of galectin-3 is normal in medium that contains serum, it is dramatically reduced in serumless medium (4). In the present study, we have exposed breast carcinoma cells to different ECM proteins in order to identify the ECM proteins likely to elicit the release/secretion of galectin-3. Our studies demonstrate that fetuin, the serum glycoprotein abundant in fetal blood, if added to serumless medium in concentrations similar to that in medium supplemented with 10% fetal bovine serum, is capable of releasing intracellular galectin-3 rapidly from breast carcinoma cells. Fetuin was previously shown to bind to various tumor cells and to induce cell aggregation by binding to lectin-like molecules (6). More recently, it was demonstrated that insect cells that express galectin-3 on their surfaces undergo homotypic aggregation in the presence of asialofetuin or fetuin (5). It is, therefore, possible that fetuin can interact with cell surface lectins and that this is sufficient signal to trigger the release of galectin-3 and possibly other members of the family. Galectin-3 is released rapidly from intracellular domains in a dose-dependent manner. Changes in intracellular calcium ion concentration do not influence the release that appears to be mediated by a novel mechanism. The data further suggest that the galectin-3 released by the cells is responsible for the rapid adhesion and spreading of breast carcinoma cells to the substrata.

MATERIALS AND METHODS

Human breast epithelial cell lines MDA-MB-435; BT-549; and 11-9-1-4, which is a galectin-3-transfected BT-549, were kindly donated to us by Dr. Avraham Raz, Karmanos Cancer Institute, Detroit, MI. All of the cell lines were cultured in DMEM/F12 (Sigma) supplemented with 100 μg/ml penicillin-streptomycin, 2.5 μg/ml Fungizone, 20 ng/ml epidermal growth factor, 98 ng/ml cholera toxin, 10% heat-inactivated fetal bovine serum, 2 mM glutamine and nonessential amino acids. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Human ECM was purchased from Collaborative Research and all of the other biochemicals from Sigma Chemical, unless otherwise stated.

Rapid Release of Galectin-3 from Cells. To assay for galectin-3 release, the cells were removed from the culture flasks by trypsinization and washed twice in serumless DMEM/F12 medium. After the last wash, the cells were left standing in suspension in the centrifuge tubes for at least 10 min. The cells were then counted using a hemocytometer and 500,000 cells in approximately 20 μl of serumless medium added to siliconized Eppendorf tubes containing 100 μl of serumless DMEM/F12 with the various additives. In the first experiment, the additives were: (*a*) fibronectin (2 mg/ml); (*b*) collagen IV (2 mg/ml); (*c*) human ECM (2 mg/ml); (*d*) TDG (100 mM); (*e*) lactose (100 mM); (*f*) 10% serum; and (*g*) 0.25% fetuin. The samples were incubated for 10 min at room temperature and centrifuged to pellet the cells, and the supernatant from each tube (20 μl) was assayed for galectin-3 by Western blot as described previously (6). The galectin-3 release assay was repeated with different doses of fetuin (0–1%) and with 0.25% fetuin to obtain a time-course of release.

The Source of Released Galectin-3. To demonstrate that the secreted fetuin is from intracellular domains and not from the cell surface, cell surface proteins were labeled with biotin, and the labeled proteins were then chased after treatment of the cells with fetuin. Briefly, cells in 75-cm^2 culture dishes were washed with serumless medium five times and then with PBS two times. The cells were then incubated with 2.6 mM NHS-Biotin (Biorad) for 30 min at room temperature with occasional swirling. The unreacted biotin was removed and the flasks washed thoroughly with PBS, and then trypsinized. Trypsin was inactivated by the addition of complete medium containing serum, and the cells were centrifuged. The cells were washed twice with serumless medium and then divided into two Eppendorf tubes (500,000 cells/tube) in 100 μl of serumless medium without (control) or with 0.25% fetuin. The cells were then incubated for 30 min at 37°C and centrifuged; 20 μl of the supernatant were taken from each tube (conditioned medium) and applied to SDS-PAGE gel.

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[3] The abbreviations used are: ECM, extracellular matrix; TDG, thiodigalactoside; CRD, carbohydrate recognition domain.

The cell pellets were lysed in lysis buffer in the presence of protease inhibitors, and the membrane fractions were subjected to SDS-PAGE. The gels were blotted onto nitrocellulose and incubated with avidin-peroxidase followed by chemiluminescence reagents as described previously (7).

Effect of Fetuin on Intracellular Calcium Levels in Breast Cancer Cell Lines. Cells were trypsinized and washed in Krebs-Ringer-HEPES buffer (118.5 mM NaCl, 4.74 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 2.54 mM $CaCl_2$, 24.9 mM $NaHCO_3$, 10 mM glucose, and 0.03 mM EDTA). They were then loaded with 4 μM of fura-2/AM in the same buffer for 45 min. At the end of the incubation, the cells were washed and placed in a cuvette with stirrer, and fluorescence measurements were made using a SPEX dual wavelength (AR-CM) fluorometer. After about 100 s of stabilization, fetuin was added to a final concentration of 0.25% and changes in intracellular calcium ion concentrations were monitored.

Effects of Calcium Ionophore A23187 and Thapsigargin in the Rapid Release of Galectin-3 from Breast Carcinoma Cells. To determine whether agents that increase intracellular calcium could induce the rapid release of galectin-3, the assay was done in the absence or presence of 5 μM of A23187 in serumless DMEM/F12 containing 1 mM $CaCl_2$. The assay was also done in the presence of 5 μM of A23187 and 0.25% fetuin. Thapsigargin (1 μg/ml), a known inhibitor of the endoplasmic reticulum Ca^{2+} ATPase was also tested in the assay by itself and in the presence of 0.25% fetuin.

Role for the Released Galectin-3 in Cell Spreading and Adhesion. We have previously demonstrated that galectin-3-expressing cells adhere and spread rapidly to ECM proteins compared with galectin-3 null-expressing cells (7, 16). In all of these assays, however, medium containing 10% serum was used. We, therefore, questioned whether galectin-3 release by fetuin was sufficient for this rapid adhesion and spreading. The cells were trypsinized and washed in serumless medium as described above. They were then allowed to adhere to tissue culture microtiter wells in the presence or absence of 0.25% fetuin in serumless DMEM/F12 containing Ca^{2+} ions. The adhesion assay was also done in the presence of 0.25% fetuin and 100 mM TDG. As a negative control (lack of rapid adhesion and spreading), BT-549 cells that do not express galectin-3 were also allowed to adhere in the presence of 0.25% fetuin. The cells were allowed to adhere for 30 min and then were photographed by a digital camera, and the images analyzed by adobe photoshop.

Adhesion of Breast Carcinoma Cells to Elastin in the Absence and Presence of Fetuin. To further implicate galectin-3 in cell-to-ECM interactions, we questioned whether the fetuin-induced rapid release of the lectin from the cells could ligate galectin-3 producing cells to elastin. Elastin (40 mg) was treated with anhydrous hydrazine and 1% hydrazine sulfate for 5 h at 80°C until all of the elastin was dissolved. The solution was then diluted with PBS to a final concentration of 2 mg/ml. The wells of a microtiter plate were then coated with the solubilized elastin for 1 h at 37°C. Nonspecific sites were blocked with 2% BSA, and cells were added to the wells in DMEM/F12 serumless medium without divalent ions and with or without 0.25% Fetuin. The cells were allowed to adhere for 12 h, and the nonadherent cells were washed off in the serumless medium. The adhered cells were photographed, and the number of cells adhered were estimated by the methylene blue assay (17).

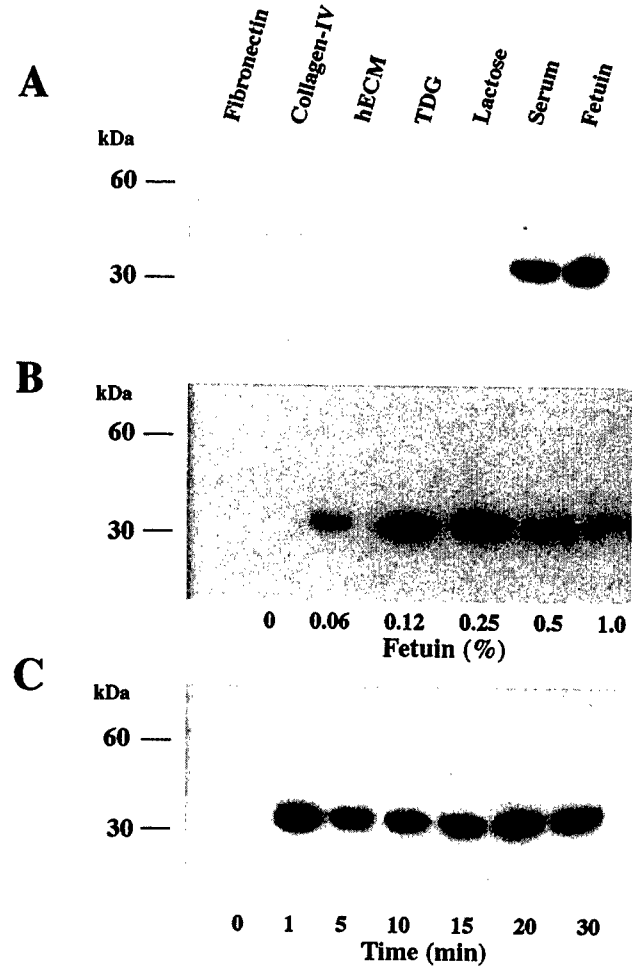

Fig. 1. *Fetuin-mediated release of galectin-3 from MDA-MB-435 breast carcinoma cells into the medium.* In *A*, the cells (500,000 cells/tube) were exposed to the ECM proteins, β galactoside sugars, serum, and fetuin in serumless DMEM/F12 for 10 min. and released galectin-3, assayed by Western blot. In *B*, the cells were exposed to various concentrations of fetuin for 10 min and galectin-3 was assayed. In *C*, the cells were exposed to 0.25% fetuin for the stated time points, and galectin-3 was assayed. *kDa*, M_r in thousands.

RESULTS

Serumless medium in our hands consistently fail to show secreted galectin-3, at least when the blots were exposed to X-ray films in less than 10 min, as we routinely did in this report. As shown in Fig. 1*A*, only fetuin and DMEM/F12 medium supplemented with 10% fetal bovine serum (complete) was able to trigger the release of galectin-3 into the medium. Fetuin was used at a concentration of 0.25% (2.5 mg/ml), which is comparable to the concentration of fetuin in the complete DMEM/F12 medium. From the level of galectin-3 released in both cases, the data suggest that fetuin was the ingredient in DMEM/F12-10% FBS that was responsible for the release of the lectin. Galectin-3 was also released from MDA-MB-435 and 11-9-1-4 by human fetuin, α 2HS glycoprotein (2 mg/ml) (data not shown). It is evident from Fig. 1*B* that the release of galectin-3 is dependent on the dose of fetuin used. Whereas the concentration of fetuin in the fetal blood can be as high as 20 mg/ml, the level drops to approximately 0.6 mg/ml in the adult (18). According to the dose-response data, fetuin levels in both fetus and adult are able to induce the release of galectin-3. The present data suggest that one possible function of fetuin *in vivo* is to trigger the release of intracellular galectin-3, at least in tumor cells. The induction of galectin-3 release by fetuin is very rapid, taking place within 1 min of exposure of cells to fetuin (Fig. 1*C*). Prechilling the cells at 4°C prior to adding fetuin did not affect the release of galectin-3 (data not shown), which demonstrates that the release was not affected by temperature. Inclusion of methylamine or propylamine in the fetuin medium did not change the level of galectin-3 released (data not shown), which implies that the pathway is independent of the endocytosis/exocytosis (4).

The Source of Galectin-3 Released into the Medium. It can be argued that galectin-3 that is rapidly secreted into the medium on fetuin stimulation is cell-surface bound and is not from the intracellular milieu. We, therefore, biotynilated the surface proteins of the breast carcinoma BT-549 clone 11-9-1-4 cells. As evidenced in Fig. 2, all of the label was retained in the cell membrane (Fig. 2, *Lanes 1* and *2*). The conditioned medium in neither the control (Fig. 2, *Lane 3*) nor the fetuin-treated (Fig. 2, *Lane 4*) tubes revealed any protein band after the normal 1–10-min exposure of X-ray film to the immunoblot membrane. Overnight exposure of the film revealed faint bands in

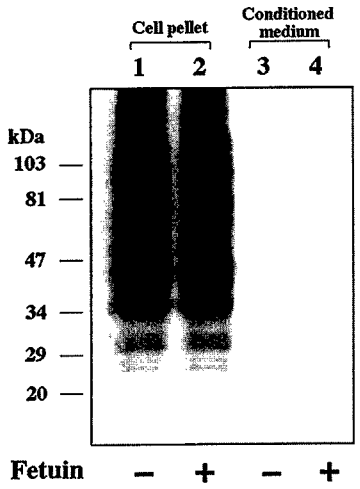

Fig. 2. *Cell surface is not the source of released galectin-3.* The cells (11-9-1-4) were biotynilated and washed, and the cells were incubated without or with fetuin in serumless medium for 30 min. Aliquots of the cell pellet fraction and conditioned medium were run through SDS-PAGE, transferred to immobilon, and the membrane incubated with avidin-peroxidase and chemiluminescent reagents and exposed to X-ray film as described in "Materials and Methods." All of the label was retained in the cell pellet fraction. *kDa*, M_r in thousands.

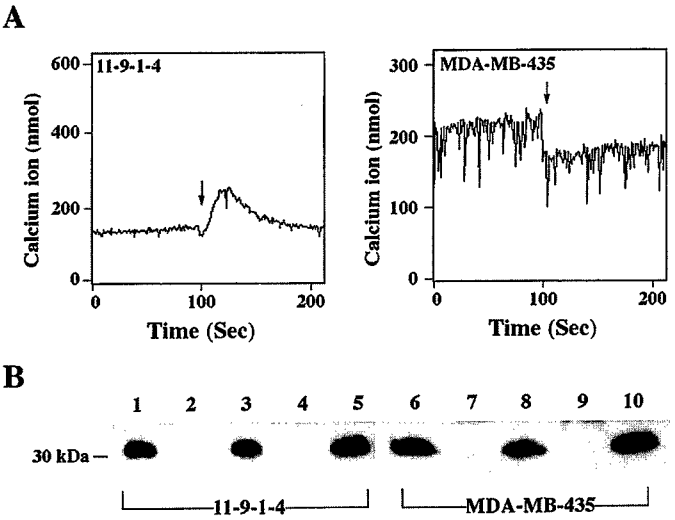

Fig. 3. *Intracellular calcium ion concentration changes and release of galectin-3.* In *A*, intracellular calcium ion changes were determined after the addition of 0.25% fetuin (*arrow*) to fura-2/AM-loaded breast carcinoma cells. The experiment was repeated three times with similar results. In *B*, 11-9-1-4 and MDA-MB-435, were each treated with the following reagents in serumless DMEM/F12 containing 1 mM Ca^{2+}, and galectin-3 was assayed as described: 0.25% fetuin (*Lanes 1* and *6*); calcium ionophore A23187 (5 μM) (*Lanes 2* and *7*); a combination of A23187 and fetuin (*Lanes 3* and *8*); thapsigargin (1 μg/ml) (*Lanes 4* and *9*); and a combination of thapsigargin and fetuin (*Lanes 5* and *10*). *kDa*, M_r in thousands.

both the control and fetuin-treated conditioned medium. Moreover, all of these bands were above M_r 40,000 (data not shown).

Intracellular Calcium Ion Concentration in Fetuin-induced Galectin-3 Release. To investigate the mechanism(s) by which fetuin affects the release of galectin-3, we analyzed intracellular calcium concentration in the breast carcinoma cells before and after the addition of fetuin. Increases in intracellular calcium ion concentration have been shown to stimulate galectin-3 secretion (4, 12). As can be seen in Fig. 3*A*, fetuin raised the intracellular calcium in 11-9-1-4 transiently by about 30%. However, in the MDA-MB-435 cell line, the addition of fetuin actually reduced intracellular calcium levels (Fig. 3*A*). We also challenged the cells with agents that are known to increase intracellular Ca^{2+} ion concentration in the absence and presence of 0.25% fetuin. Galectin-3 was released from both 11-9-1-4 and MDA-MB-435 carcinoma cells in the presence of fetuin (Fig. 3*B*, *Lanes 1* and *6*, respectively). The inclusion of A23187 in serumless medium (Fig. 3*B*, *Lanes 2* and *7*) failed to release galectin-3. The inclusion of A23187/fetuin (Fig. 3*B*, *Lanes 3* and *8*) in the serumless medium triggered the release of galectin-3 in levels comparable with controls. Similarly, thapsigargin, the inhibitor of endoplasmic reticulum ATPase, by itself failed to trigger the release of galectin-3 (*Lanes 4* and *9*) and did not enhance the release in the presence of fetuin (*Lanes 5* and *10*). Taken together, the data suggest that fetuin-induced release of galectin-3 is independent of changes in intracellular calcium.

Rapid Cell Spreading and Adhesion Modulated by Galectin-3 Released from Cells by Fetuin. We show here that the 11-9-1-4 breast carcinoma cells spread and adhere to microtiter wells very rapidly in serumless medium containing fetuin (Fig. 4*A*) compared with adhesion in the absence of fetuin (Fig. 4*B*). The rapid adhesion and spreading in the presence of fetuin was slowed down considerably in the presence of 100 mM TDG (Fig. 4*C*). The parental BT-549 cells that do not express galectin-3 failed to spread and adhere in the presence of fetuin (Fig. 4*D*), requiring an overnight incubation to display cell spreading. The data demonstrate that fast spreading and adhesion to tissue culture plates requires intracellular galectin-3 released rapidly by fetuin. The failure of parental BT-549 to spread and adhere quickly suggests that galectin-3 and not galectin-1 is the relevant lectin. The parental BT-549 cells express high levels of galectin-1 (16).

Breast Carcinoma Cells Adhere to Elastin in the Presence of Fetuin. We have previously demonstrated that the interaction of breast carcinoma cells with elastin is heavily dependent on galectin-3 expression (8). In fact galectin-3 binds specifically to elastin and is associated with tropoelastin in breast carcinoma cells, which suggests that this interaction is physiologically relevant (8). The BT-549 (galectin-3 null-expressing cells) adhered poorly to elastin in the absence

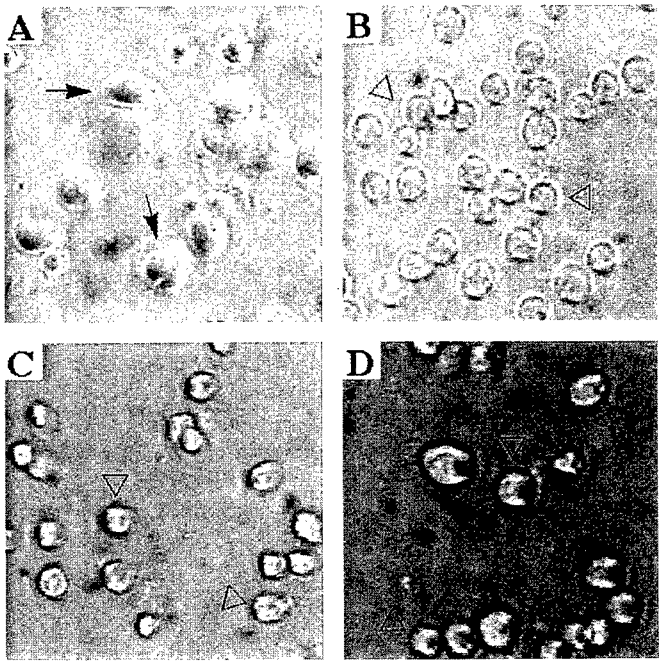

Fig. 4. *Control of rapid cell spreading and adhesion by fetuin.* The 11-9-1-4 cells (galectin-3 expressing) were plated in the wells of a microtiter plate in the presence (*A*) or absence (*B*) of 0.25% fetuin in serumless DMEM/F12 containing 1 mM Ca^{2+}. The cells were also plated in the presence of both fetuin and 100 mM TDG (*C*). As a control, BT-549 (no galectin-3) were plated in the presence of fetuin (*D*). The cells were allowed to adhere for 30 min. and then photographed by a digital camera (phase contrast).

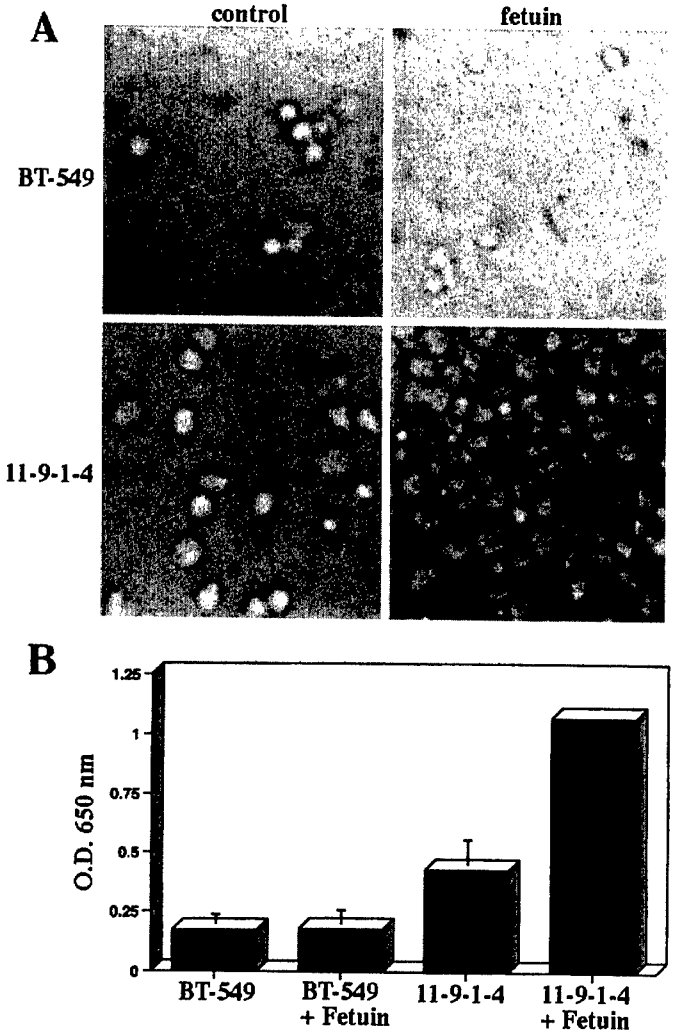

Fig. 5. *Ligation of breast carcinoma cells to elastin.* In *A*, the wells of a microtiter plate were coated with hydrazine-solubilized elastin. After blocking nonspecific sites with 2% BSA, the cells (5×10^4 cells/well) were plated in quadruplicates and were allowed to adhere to the wells in serumless DMEM/F12 medium without divalent ions and without (control) or with 0.25% fetuin and for at least 12 h at 37°C. The nonadherent cells were washed twice and the adherent cells were photographed. In *B*, after photographing the cells, they were fixed in methanol and the cell number determined. *Columns*, represent average number of cells/well. *O.D.*, absorbance.

(control) or presence of 0.25% fetuin as was expected (Fig. 5, *A* and *B*). The 11-9-1-4 cells (galectin-3 expressing), on the other hand, adhered very well to elastin in the presence of fetuin. The interaction was significantly better when compared with adhesion in the absence of fetuin (Fig. 5, *A* and *B*). The data suggest that galectin-3 released by fetuin may be directly used to ligate the breast carcinoma cells to elastin-rich tissues such as the lungs.

DISCUSSION

In this report, we have demonstrated that fetuin, a serum glycoprotein, is capable of eliciting the rapid release of galectin-3 from breast carcinoma cells in a novel fashion. The data address a fundamental problem in biology, namely the role of fetuin and galectin-3 in cell growth regulation. The elucidation of the nonclassical pathway of secretion of galectin-3 is critical in order to understand its precise physiological role in cell-to-ECM interactions. Galectins, particularly galectin-1 and -3, have long been suspected of regulating the adhesion of a wide variety of cell types to ECM (19). We have previously demonstrated that galectin-3 plays a crucial role in the plating and cloning efficiencies of breast carcinoma cells. The cells that express galectin-3 interact more efficiently with substrata such as laminin, collagen IV, elastin, and soft agar compared with galectin-3 null-expressing cells (7, 8, 20). It is well documented that the galectin-3 gene is involved in tumorigenesis (6, 21, 22) and metastasis particularly of the breast and colon carcinomas (16, 23). More recently, it was reported that the sera of patients with breast cancer, gastrointestinal cancer, lung cancer, ovarian cancer, melanoma, and non-Hodgkin's lymphoma had significantly higher levels of galectin-3, compared with sera of normal subjects (24). Moreover, galectin-3 concentrations in sera from patients with metastatic disease were higher than in sera from patients with localized tumors (24). This study suggests that circulating galectin-3 plays a role in tumor progression and that fetuin could be a player in the secretion of galectin-3 into the sera.

The rapid release of galectin-3 on contact of the cells with fetuin and not other ECM proteins is interesting because fetuin and its desialylated form (asialofetuin) interact strongly with galectin-3 via the CRD of the lectin. Normally galectin-3 is expressed on the cell surface presumably bound via its CRD to glycans containing polylactosamine residues such as lysosomal-associated membrane proteins (25). Galectin-3 molecules can also interact with each other via their NH_2-domains, freeing extra CRD domains to interact with other glycoproteins such as laminins in ECM. We, therefore, suspected that the interaction of cells with glycans such as laminin and collagen IV, which have polylactosamine residues, may trigger the release of more galectin-3 from intracellular stores. The present data demonstrate that this is not the case for any of the glycans examined except fetuin. It is, therefore, apparent that the pathway by which fetuin triggers the release of galectin-3 from the intracellular stores is novel. Previous studies demonstrated clearly that serum is essential for galectin-3 secretion. Apart from fetuin, which is the major serum protein in bovine fetal blood, serum contains numerous proteins. In as much as the present study does not rule out all the other serum proteins in the process of galectin-3 release from intracellular stores, fetuin appears to be the critical factor in serum for the externalization of galectin-3 and hopefully other members of the family.

There are a number of models that have been suggested for galectin-3 secretion. For example, it has been proposed that before secretion, galectin-3 accumulates at sites on the cytoplasmic side of plasma membranes (12–14). This step of accumulation is rate limiting and can be up-regulated by heat shock and calcium ionophores (4, 13, 26, 27). The next step in galectin-3 secretion appears to be evagination of plasma membrane, a process that requires NH_2-terminal domains of the protein (15). Finally, the process consists of the pinching off of evaginating plasma membrane domains and the release of galectin-3 from the externalized vesicles. However, as has been noted by others, some galectin-3 molecules may be released from plasma membrane domains directly into the extracellular medium (15). It is this pathway that appears to be supported by our data, because we are defining a process that takes place extremely rapidly (within minutes) and is mediated by fetuin. The molecular mechanisms of this pathway may involve other proteins such as chaperons. Our data clearly demonstrate that galectin-3 is from the cytoplasmic domains and not from the cell surface. The fetuin may induce the secretion of galectin-3/chaperon complex, thereby modulating the last stages of the externalization process and not the rate-limiting step.

On the basis of the data, we propose that the released galectin-3 is immediately recruited to modulate cell spreading and adhesion to the substratum. Galectin-3 may do this by interacting with and activating cell surface adhesion molecules and cytoskeleton elements via its CRD domains because this interaction is abrogated by TDG. Cells that lack galectin-3 but express galectin-1, such as BT-549 breast carci-

noma, lack this rapid cellular adhesion and spreading, as observed previously (7). Interestingly, fetuin has been implicated in cell spreading, stretching, and adhesion in other cell systems (18). The rapid cellular adhesion and spreading that is catalyzed by fetuin and galectin-3 may well explain cell-growth-promoting activities of fetuin (18). The cells that have the capacity to adhere and spread quickly to substrata obviously will have a growth advantage over those that spread and adhere more slowly. We recently demonstrated that the interaction of breast carcinoma cells with elastin could be directly linked to galectin-3 expression because exogenously supplied galectin-3 was able to ligate these cells to elastin. Galectin-3-expressing cells interacted well and proliferated on elastin fibers, but only in the presence of complete medium containing serum (8). We now show that fetuin-mediated release of galectin-3 in serumless DMEM/F12 is sufficient to ligate galectin-3-expressing cells to elastin whereas galectin-3 null-expressing cells are not ligated. The ligation occurred over a 12-h period, because a critical galectin-3 concentration has to be achieved for the adhesion to occur.

It can be argued that the fetuin-induced galectin-3 release is not necessary for *in vivo* cell growth and differentiation, because fetuin-deficient mice are fertile and mature normally (28). This suggests that there are other proteins or growth factors apart from fetuin that may trigger the rapid release of galectin-3. Alternatively, rapid release of galectin-3 may be relevant only in tumor cells, in which it confers a growth advantage. Similarly, galectin-3-null mutant mice are viable with no abnormalities (29). In this case, other members of the galectin family, such as galectin-5, are likely to substitute for galectin-3. Decrease or lack of tumorigenicity or metastatic potential in either fetuin- or galectin-3-deficient mice would be an interesting observation.

In summary, fetuin can induce a very rapid release of galectin-3 from breast carcinoma cells. This release takes place within 1 min and is necessary for the activation and modulation of cell surface receptors for ECM proteins. The released galectin-3 can also be used to ligate breast carcinoma cells to elastin rich tissues such as lungs during the metastatic dissemination of breast cancer. The novel pathway by which galectin-3 is released is independent of changes in intracellular calcium and temperature. The galectin-3 is most likely released from vesicles close to the plasma membrane. The release is not influenced by factors that normally affect the exocytosis or endocytosis pathways. The present data suggest the mechanism(s) by which fetuin may modulate the cellular adhesion and growth of breast epithelial cells *in vitro* and *in vivo*.

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